REMARKS

This is in response to the Office Action that was mailed on January 17, 2001. Claims 1-9 are in the case.

The sequence identifiers in the specification have been amended as required by the Examiner.

Claims 1-7 were rejected under the second paragraph of 35 USC 112, the Examiner questioning the definiteness of the term "substantially" in the phrase "substantially not immunointeractive with human sera". One skilled in the art would have no difficulty in determining whether a particular polypeptide was substantially not immunointeractive with human sera in the sense of the present claims. In any case, Applicants provide extensive guidance for determining the substantial absence of immunointeractive, for instance in the specification, from line 10 on page 22 through line 29 on page 24. See also Example 8 on pages 34-36 of the specification. In order to further clarify this point, Applicants present in the IMMUNO-REACTIVITIES APPENDIX extensive data relating thereto. Finally, Applicants also enclose a copy of their article "Cloning and expression of Immunoreactive Antigens from *Mycobacterium tuberculosis*", Clinical and Diagnostic Laboratory Immunology, July 2000, pp. 600-606. It is respectfully submitted that – considering the level of skill of the persons for whom the present application is written – the claims in their present form satisfy the requirements of the statute.

Claims 8 and 9 were rejected under the second paragraph of 35 USC 112, due to their form of identifying sequences. The claims have been amended to conform to current USPTO practice, thus obviating the rejection.

Claims 1-7 were rejected under 35 USC 102(b) as being anticipated by the Thybo article. The rejection is respectfully traversed. Thybo is concerned with serodiagnosis as such. The present invention provides new markers for use in serodiagnosis of TB. Thus, the present claims relate to the immuno-reactivity of antigens for TB, whereas the aim of Thybo's study was "to evaluate the value of serodiagnosis of TB by ELISA in an HIV and TB endemic region" (p. 153). In any case, as can be seen from the "totals" rows in Tybo, the controls had antigen titers averaging 0.70 (0.68 + 0.72 / 2) as compared to antigen titers for Tybo's polypeptide averaging 1.15 (1.03 + 1.26 / 2). An antigen titer of 0.70 does not meet the present claims' requirement "which polypeptide is substantially not immunointeractive with sera from a human, animal or avian species not prior exposed to said species of *Mycobacterium* or its relative or its antigenic parts". Accordingly the present claims are not anticipated by Thybo.

Conclusion

It is believed that a full and complete response has been made to the Office Action, and that as such, the Examiner is respectfully requested to send the application to Issue.

In the event there are any matters remaining in this application, the Examiner is invited to contact Mr. Richard J. Gallagher, Registration No. 28,781 at (703) 205-8000 in the Washington, D.C. area.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By: Richard Gallagher 28,781
for Gerald M. Murphy, Jr.
Reg. No. 28,977

GMM/RG/clb

P. O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

Enclosures: Marked Up Version of Amendments
Immuno-reactivities appendix
Lim et al. article

MARKED UP VERSION OF AMENDMENTS

IN THE SPECIFICATION:

Please cancel the paragraph bridging pages 2-3.

Please amend the paragraph in lines 12-14 on page 4 to read:

Yet a further aspect of the present invention provides a polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or an amino acid sequence having at least 60% similarity to any one of said sequences.

Please amend the paragraph in lines 15-19 on page 4 to read:

Still yet a further aspect of the present invention provides a polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or a[n] nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Please amend the paragraph in lines 25-27 on page 10 to read:

The present is particularly exemplified in relation to *Mycobacterium* antigens B.4, B.6, B.10, MMP and C17 having amino acid sequences and

corresponding nucleotide sequence as set forth in [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 respectively.

Please amend the paragraph in lines 1-3 on page 11 to read:

Accordingly, another aspect of the present invention provides a polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 or an amino acid sequence having at least 60% similarity to any one of said sequences.

Please amend the paragraph in lines 4-7 on page 11 to read:

Yet a further aspect of the present invention provides a polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a[n] nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Please amend the paragraph in lines 8-11 on page 1 to read:

Even yet a further aspect of the present invention provides a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a[n]

nucleotide sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

Please amend the paragraph in lines 16-21 on page 36 to read:

Nucleotide sequences and corresponding amino acid sequences were determined for antigens B.4, B.6, B.10, MMP and C17 and are shown in [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 respectively.

IN THE CLAIMS:

Please cancel claims 10-26, without disclaimer of their subject matter or prejudice to reassertion in this or a continuing application.

Please amend claims 1-9 to read:

1. (amended) An isolated polypeptide or a derivative, homologue, analogue, or functional equivalent thereof wherein said polypeptide is obtainable from a species of *Mycobacterium* and which polypeptide is immunointeractive with sera from a human, animal or avian species exposed to said species of *Mycobacterium* or its relative or antigenic parts thereof but which polypeptide is substantially not immunointeractive with sera from a

human, animal, or avian species not [prior] previously exposed to said species of *Mycobacterium* or its relative or its antigenic parts.

2. (amended) The [An] isolated polypeptide according to claim 1 wherein the species of *Mycobacterium* is selected from *Mycobacterium* [is *M.*] *tuberculosis, Mycobacterium avium, Mycobacterium microti, Mycobacterium leprae, Mycobacterium lepraemurium, Mycobacterium paratuberculosis, Mycobacterium ulcerans, Mycobacterium marinum, Mycobacterium smegmatis, Mycobacterium intracellulare, Mycobacterium xenopi, Mycobacterium chelonei, Mycobacterium fortuitum, Mycobacterium farcinogenes, Mycobacterium flavum, Mycobacterium haemophitum, Mycobacterium kansasii, Mycobacterium phlei, Mycobacterium scrofulaceum, Mycobacterium senegalense, Mycobacterium simiae, Mycobacterium thermoresistible,* and *Mycobacterium xenopi.*

3. (amended) The [An] isolated polypeptide according to claim 2 wherein the species of *Mycobacterium* is *M. tuberculosis.*

4. (amended) An isolated polypeptide or a derivative, homologue, analogue, or functional equivalent thereof wherein said polypeptide is obtainable from *M. tuberculosis* or a related organism and which polypeptide is immunointeractive with sera from a human previously exposed to *M. tuberculosis* or an antigenic extract therefrom but is substantially not

immunointeractive with human sera not previously exposed to *M. tuberculosis* or an antigenic extract thereof.

5. (amended) The [An] isolated polypeptide according to claim 4 wherein the human exposed to *M. tuberculosis* has active pulmonary or extra-pulmonary tuberculosis.

6. (amended) The [An] isolated polypeptide according to claim 4 or 5 wherein the polypeptide has a [molecule] molecular weight of from about 5 kDa to about 100 kDa.

7. (amended) The [An] isolated polypeptide according to claim 6 wherein the molecular weight is selected from about 10 to 20 kDa, 28 to 38 kDa, 38 to 48 kDa, 53 to 63 kDa, and 55 to 65 kDa.

8. (amended) An isolated polypeptide comprising an amino acid sequence selected from [<400>2, <400>4, <400>6, <400>8, <400>10] SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or an amino acid sequence having at least 60% similarity to any one of said sequences.

9. (amended) An isolated polypeptide encoded by a nucleotide sequence selected from [<400>1, <400>3, <400>5, <400>7, <400>9] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or a[n] nucleotide

sequence having at least 60% similarity to any one of said sequences or a nucleotide sequence capable of hybridizing to any one of said sequences under low stringency conditions at 42°C.

IMMUNO-REACTIVITIES APPENDIX

Exp rim ntal Background

Sera Specimens. A total of 427 human sera specimens were used in this study. Control groups consisted of 178 healthy individuals, 29 individuals with non-TB respiratory disease (ie. lung cancer and asthma), and 109 individuals with inactive TB. All the inactive TB patients exhibited tuberculin skin test positive (PPD +ve), and acid fast stain (AFB) of sputa and bacteria culture negative. The test group consisted of a total of 111 sera specimens from patients with active TB confirmed either by chest X-ray or bacteriologically by culture or sputum AFB smear tests. The extrapulmonary TB sera were confirmed by histological analysis and/or bacteriological culture. These sera specimen were also tested against 2 commercially available TB diagnostic kits, T1 and T1. All sera were aliquoted and stored at -70°C until use.

Immunoblot analysis of recombinant antigens. Western blots were prepared by the conventional SDS-PAGE electrophoresis on Tris-HCl 2D preparative ready-gels (BioRad, Hercules, CA) and subsequently the electrophoresed antigen (10 μg per gel) were Western blotted onto Hybond™-C nitrocellulose membrane (Amersham Life Science, Little Chalfont, United Kingdom) as described previously. After transfer, the membranes were blocked in 5% skimmed milk/TBST, air-dried and stored at 4°C until further use.

The membranes were cut into 3 mm wide strips for testing against the TB sera panels. Screening was carried out in slot trays containing 1 ml of diluted serum specimen (1:100 in 1% skimmed milk/TBST [10 mM Tris, pH 7.5, 300 mM NaCl, 0.005% Tween 20]) per lane, for 1 h with rocking at room temperature (18-25°C). The strips were then washed 4X in TBST followed by incubation with alkaline phosphatase conjugated Goat anti-human Ig (Harlan Sera Lab, Loughborough, United Kingdom) (1:1000 in 1% skimmed milk/TBST) for 1 h with rocking at room temperature. The strips were again washed in TBST (4X), allowed to develop in 1 ml of NBT/BCIP substrate (BioRad) for 4 min and subsequently, the reaction was stopped by washing in ddH_2O (4X). Positive controls consisted of a strip probed with a positive serum specimen, reactive to the recombinant protein antigens, and a second strip probed with the commercially available anti-RGSHis probe (Qiagen). Reactivity of recombinant protein to sera specimens was interpreted based on the density of band obtained on a densitometer, X-Rite® 400 (measuring range 0.00D-2.5D; X-Rite Inc., Grandville, MI).

ELISA assay. Microtiter plates (Medium binding type II EIA strip plate; Corning Costar Corporation, Corning, NY) were coated overnight at room temperature with 100 μl/well of diluted antigen in 0.05 M sodium carbonate buffer, pH 9.6. The optimized coating concentrations per well for the antigens, Antigen Seq. ID

2, 4, 6, 8, 10 and 38-kD were 0.05, 0.065, 0.025, 0.10, 0.025 and 0.029 μg respectively. After two washes with wash buffer [0.05% Tween 20 in phosphate-buffered saline (PBS), pH 7.4], the coated plates were blocked with 200 μl/well of blocking buffer [1% bovine serum albumin (BSA) (Sigma, St Louis. Mo), 2.5% sucrose (Sigma) in sodium carbonate buffer] for 2 h in room temperature. After incubation, the plates were emptied, dried and subsequently stored at 2-8°C until further use.

For the assay, 100 μl diluted (1:51) sera specimen with the sample diluent, 1:25 v/v normal Goat Serum (Gibco BRL Life Technologies, Inc., Grand Island, NY), 0.05% Tween 20 (Sigma), 0.02% Thimerosal (Sigma) and 0.3 ml/L phenol (Sigma) in PBS pH 7.4, was added to each well and incubated either for 20 min in room temperature (Seq. ID 8 and 10) or for 1 h in 37°C (Seq.ID 2, B.4, B.6 and 38-kD). After incubation, the wells were washed 3X with 200 μl wash buffer (0.05% Tween 20, 0.02% Thimerosal and 0.3 ml/L phenol in PBS pH 7.4). In the following step, the wells were filled with 100 μl of diluted (1:5,000 for Seq. ID 2; 1:6,000 for Seq. ID 4; 1:8,000 for Seq. ID 6 and 8; 1:10,000 for Seq.ID 10; and 1:5,000 for 38-kD antigen) Horseradish peroxidase-conjugated Goat Anti-Human IgG, Fc_γ fragment specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in a conjugate diluent [1:4 (v/v) Fetal Bovine Serum (Gibco BRL), 1:10 (v/v) Glycerol (Sigma), 0.05% Tween 20, 0.02% Thimerosal and 0.3 ml/L phenol in PBS pH 7.4] and incubated either for 20 min in room temperature (Seq, ID 8 and 10) or for 30 min in 37°C (Seq.ID 2, 4, 6 and 38-kD). The wells were washed 3X after which 100 μl of one-step TMB (3,3', 5,5'-tetramethylbenzidine) substrate (Dako Corporation, Carpinteria, CA) was added to each wells followed by incubation either for 10 min in room temperature (Seq. ID 8 and 10) or for 15 min at 37°C (Seq. ID 2, 4, 6 and 38-kD). The enzymatic reaction was stopped by the addition of 100 μl of 1 N sulfuric acid. Absorbance was read within 15 min in an ELISA reader (Spectra, TECAN Austria Ges. m.b.H, Grödig, Austria) at 450 nm with 630 nm as a reference. Blank wells, negative and positive control sera specimens were included in each plate. All the sera specimens were analyzed in duplicate.

Cutoff values. Evaluation of diagnostic specificity for the antigens were based on a positive score represented by antibody titers (interpreted as densitometer units, D, for Western blot and optical density, OD, for ELISA) above the cutoff values calculated based on the means of a control group of healthy individuals (n = 30, BioClinical). On the ELISA assay, the cutoff values were defined as mean OD plus multiples of standard deviations obtained for the control group (Table). The cutoff values were calculated individually for each antigen. With the ELISA format, the number of standard deviations above the mean OD used is shown in footnote in the table. On the Western format, the cutoff were also calculated individually for each antigen, based on the mean of density (D) observed for the 30 normal sera from Bioclinical Inc.

Commercial TB diagnostic kits. As a comparison, all the sera specimens were also screened using two commercially available TB diagnostic kits. An immuno-chromatography-based TB diagnostic kit (T1), ICT Tuberculosis (ICT Diagnostics, Brookvale, NSW, Australia) and an ELISA test kit (T2), PATHOZYME-TB Complex plus (Omega Diagnostics Limited, Alloa, Scotand, United Kingdom) were used.

Statistical Analysis. Assay sensitivities, specificities, positive and negative predictive values were calculated using the statistical software, Win Episcope 1.0 (Borland International Inc., Scotts Valley, CA). Distribution plots were generated using the GraphPad Prism 2.01 software.

The percentage of reactivity for the recombinant TB antigens with different sera specimens as observed on the Western blot and ELISA assays is shown in the Table. Also shown is the percentage of sera specimens detected positive by the commercial TB diagnostic kits (T1 & T2).

Antigen Sequence ID	Theoretical Molecular Weight	Observed Molecular Weight	Healthy Controls (n=178)	Active TB (n=111)	Inactive TB (n=109)	Lung Cancer (n=19)	Asthma (n=10)	PPV[a]	NPV[b]
(a) W stern Blot Assay									
Seq. ID 2	55.8	56±3	9.0	29.7	12.8	10.5	10	67.4	67.5
Seq. ID 4	55.0	55±3	8.4	25.2	12.8	5.3	0	65.1	66.3
Seq. ID 6	32.9	33±3	2.2	19.8	4.6	5.3	10	84.6	66.2
Seq. ID 8	16.1	16±3	2.8	27.0	21.1	0	0	85.7	68.1
Seq.ID 10	37.5	38±3	12.9	17.1	7.3	0	0	45.2	62.8
38-kD	NA	NA	16.8	44.1	31.2	10.5	10	62.0	70.5
T1*	NA	NA	7.3	63.1	44.0	10.5	0	84.3	80.1
(b) ELISA assay									
Seq. ID 2	55.8	56±3	6.7	42.3	7.3	10.5	0	79.7	72.2
Seq. ID 4	55.0	55±3	1.7	25.2	4.6	0	0	90.3	67.8
Seq. ID 6	32.9	33±3	6.2	19.0	7.3	0	0	65.6	65.0
Seq. ID 8	16.1	16±3	6.7	35.1	22.0	15.8	0	76.5	69.8
Seq.ID 10	37.5	38±3	12.4	7.2	12.8	5.3	0	26.7	60.2
38-kD	NA	NA	1.7	53.2	18.4	10.5	0	95.2	77.1
T2*	NA	NA	2.2	44.1	30.3	0	0	92.4	73.7

On the Western assay (detecting for human Ig), the cutoff values were: >0.04D for Seq. ID 2 and Seq. ID 4 band; ≥0.04D for Seq. ID 6 and Seq. ID 8 band; ≥0.15D for Seq. ID 10 band; and >0.15D for 38-kD band. On the ELISA assay (detecting for human IgG), the cutoff values were: 0.52 (Mean+4SD) for Seq. ID

2; 2.14 (Mean+3SD) for Seq. ID 4; 0.53 (Mean+4SD) for Seq. ID 6; 0.09 (Mean+2SD) for Seq. ID 8; 0.10 (Mean+3SD) for Seq. ID 10; and 1.06 (Mean+3SD) for 38-kD.

[a] PPV, positive predictive value, P >0.05; [b]NPV, negative predictive value, P >0.05

* T1 and T2 commercial kits make use of combinations of 5 and 2 different antigens, respectively.

NA= not applicable.

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 2000, p. 600–606 Vol. 7, No. 4
1071-412X/00/$04.00+0

Cloning and Expression of Immunoreactive Antigens from *Mycobacterium tuberculosis*

RENEE LAY HONG LIM,[1]* LI KIANG TAN,[1] WAI FUN LAU,[1] MAXEY CHING MING CHUNG,[1,2] ROSEANNE DUNN,[1]† HENG PHON TOO,[2] AND LILY CHAN[1]

Bioprocessing Technology Centre[1] and Department of Biochemistry,[2] The National University of Singapore, Singapore

Received 1 December 1999/Returned for modification 8 February 2000/Accepted 4 April 2000

Four immunoreactive proteins, B.4, B.6, B.10, and B.M, with molecular weights ranging from 16,000 to 58,000, were observed from immunoblots of *Mycobacterium tuberculosis* total lysates screened with sera from individuals with active tuberculosis. These proteins were identified from microsequence analyses, and genes of proteins with the highest homology were PCR amplified and cloned into the pQE30 vector for expression studies. In addition, a 37.5-kDa protein, designated C17, was identified from a phage expression library of *M. tuberculosis* genomic DNA. Preliminary immunoblot assays indicated that these five resultant recombinant proteins could detect antibodies in individuals with active pulmonary and extrapulmonary tuberculosis. The overall ranges of sensitivities, specificities, positive predictive values, and negative predictive values for the recombinant antigens were 20 to 58, 88 to 100, 69 to 100, and 56 to 71%, respectively. The B.6 antigen showed preferential reactivity to antibodies in pulmonary compared to nonpulmonary tuberculosis serum specimens. All of these recombinant antigens demonstrated potential for serodiagnosis of tuberculosis.

Tuberculosis (TB) is a major health problem in the developing world as well as a disease which is reemerging as a major health threat in the developed world (6). World Health Organization (WHO) statistics indicate that one-third of the world's population is currently infected (34). TB is rated the second most common infectious disease and has the highest mortality rate of any infectious disease in the world. Currently there are 30 million cases of active TB worldwide, with approximately 8 million new cases and 3 million deaths reported annually. In addition, 50 million people may already be infected with multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*. A high prevalence of TB is also associated with human immunodeficiency virus (HIV) infection and AIDS and is now becoming the leading cause of death among HIV-positive individuals, with a fatality rate of 80% (34).

Control of this disease revolves around good patient care and management. In particular, early detection and treatment of tuberculosis can limit transmission of the bacilli. Conventional tests for the diagnosis of tuberculosis include chest X-ray, direct sputum smear for acid-fast bacilli, culture test, and the skin tuberculin PPD (purified protein derivative) test (29). Among these, the culture method is time-consuming but reliable. PCR and nucleic acid-based methods for detecting *M. tuberculosis* DNA sequences require complex equipment and highly skilled staff, and they are expensive and unsuitable for routine diagnostic testing in developing countries (5). Rapid serological diagnostic tests such as the enzyme-linked immunosorbent assay (ELISA) and membrane chromatography tests, in contrast, are simple and inexpensive, and the latter can be point-of-care devices (3). A major problem encountered in serological techniques is the specificity and reactivity of antigens used. A majority of *M. tuberculosis* antigens studied to date have homology with analogous proteins of environmental mycobacteria or other bacteria, resulting in unspecific reactivity to antibodies in patients with inactive TB or nontuberculous infections (8, 23). Hence, positive test results produced by these known antigens are generally unreliable, and supplementary tests are required to confirm tuberculosis infection. It was shown that the use of recombinant *M. tuberculosis* antigens of specific purity or particular epitopes may enhance the specificity and sensitivity of serological testing for TB when used in a panel of recombinant antigens (1). Rapid diagnostic tests that are specific and sensitive would be useful in both seroepidemiological and clinical studies pertaining to tuberculosis control and prevention. In this report, we describe the identification, isolation, and characterization of five recombinant antigens from *M. tuberculosis* for use as serodiagnostic markers for tuberculosis.

MATERIALS AND METHODS

***M. tuberculosis* total-protein extraction.** *M. tuberculosis* cells (ATCC 27294) were cultured in MycoFlasks (Gibco, BRL) containing Lowenstein-Jensen medium at 37°C with 1.5% CO_2 in a humidified incubation chamber (Jouan IG/50 model). Confluent cells from six culture flasks were harvested by adding 3 ml of Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) into each flask and gently flushing the surface of the flask. Dislocated cells were placed into sterile plastic tubes (Falcon), and cells were pelleted by centrifugation at 1,100 × *g* for 5 min. Cells were washed once in an equal volume of distilled H_2O before being resuspended in an equal volume of distilled H_2O. The cell suspension was then heated to 90°C for 2 h and frozen at −20°C overnight. Cells were then thawed on ice and pelleted by centrifugation at 20,000 × *g* for 10 min. Extraction of total protein was performed by adding 500 μl of 8 M urea solution to 0.5 g of cell pellet, vortexing the cell suspension at room temperature for 20 min, and heating it at 90°C for 2 min. Insoluble cellular debris was removed by centrifugation at 20,000 × *g* for 10 min, and the supernatant containing the extracted total protein was kept at −20°C until further use.

Western blot analysis. The total-protein extract of *M. tuberculosis* was fractionated on a sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) gel (20) and transferred onto a nitrocellulose membrane by Western blotting (33). Strips from the immunoblot were probed against pooled positive (tested positive by skin PPD and culture) and negative sera from nine individuals with active TB and seven healthy individuals, respectively. Incubation with pooled sera (1:100 in 1% skim milk-TBST [10 mM Tris, pH 7.5, 300 mM NaCl, 0.005% Tween 20]) was carried out with rocking for 1 h at room temperature. The blots were then washed four times in TBST before incubation with alkaline phosphatase–conjugated goat anti-human immunoglobulin (Ig) (Harlan Sera Lab, Loughborough, United Kingdom) (1:1000 in 1% skim milk-TBST) for another 1 h. The strips were again washed four times in TBST followed by

* Corresponding author. Mailing address: Bioprocessing Technology Centre, National University of Singapore, 5th Floor, MD11, 10 Kent Ridge Crescent, Singapore 119260. Phone: 65-874-6222. Fax: 65-775 4933. E-mail: btclimr@nus.edu.sg.

† Present address: Department of Cell and Molecular Biology, University of Technology, Sydney, NSW, Australia.

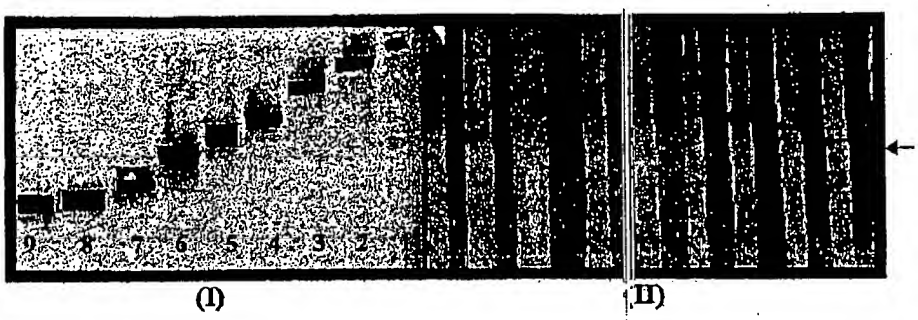

FIG. 1. (I) Gel-purified and concentrated *M. tuberculosis* protein antigens (1, 2, 3, 4, 5, 6, 7, 8, and 9) blotted onto PVDF membranes were excised for N-terminal sequencing. (II) These protein antigens were blotted onto nitrocellulose membranes and immunoscreened against pooled normal (N) and active-TB (A) sera, respectively. Positive bands (arrows) were observed with A but not with N.

incubation in 1 ml of bromochloroindolyl phosphate-nitroblue tetrazolium substrate (NBT-BCIP; Bio-Rad) for 4 min. The reaction was stopped by washing four times in distilled H_2O.

N-terminal sequencing. Individual protein bands (which were shown to react positively in the immunoscreening experiment) were excised from several preparative SDS–7.5% polyacrylamide gels and concentrated by reelectrophoresis (constant current of 18 mA at 8°C) on a long stacking gel (7 cm of 4% stacking gel, 5 cm of 10% resolving gel). The concentrated protein bands were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), stained with Coomassie brilliant blue R-250 (Sigma, St. Louis, Mo.), and excised for N-terminal microsequencing. The protein bands were also blotted onto Hybond-C nitrocellulose membranes (Amersham Life Science, Little Chalfont, United Kingdom) for validation by immunoscreening using the same pooled sera samples as described above.

Screening of a phage expression library. An expression library of *Eco*RI-restricted genomic DNA of *M. tuberculosis* was constructed in lambda ZAP Phage expression vector, according to the protocol by Stratagene (ZAP Express cDNA Synthesis kit manual, Stratagene Cloning Systems, 1998). The resultant library has 98% recombinants (2×10^5 PFU/μg arms) and insert sizes ranging from 0.7 to 2 kb. A lawn of XL1-MRF′ host cells infected with about 2×10^4 PFU of the phage stock was prepared on a 150-mm plate and incubated for 6 to 7 h at 42°C. The lawn was then overlaid with a Hybond-C nitrocellulose membrane (Amersham) presoaked in 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction of protein expression by further incubation at 37°C for 4 h. The plate and membrane were indexed for matching corresponding plate and membrane position. Approximately 0.4×10^6 plaques were screened, and as a negative control, a lawn of host cells infected with 2×10^4 PFU of the nonrecombinant lambda ZAP phage was used instead.

After transfer, membranes were washed twice in TBST buffer and blocked in 5% skim milk-TBST. Pooled sera from four individuals with active TB and from four healthy individuals were preabsorbed overnight, against negative-control membranes. Membranes from the expression library were incubated with the preabsorbed human sera for 2 h with rocking at room temperature, followed by three washes in TBST. A secondary antibody of alkaline phosphatase-conjugated goat anti-human Ig (diluted 1:1,000 1% skim milk-TBST) was added to the membranes and allowed to incubate for 1 h. After a final wash, colorimetric detection was performed using NBT-BCIP substrate as described previously. Positive plaques were cored out, and recombinant phage was eluted in SM buffer containing 2% chloroform (Stratagene manual). These were replated at about 100 to 200 PFU on 82-mm plates for secondary and tertiary screenings using the same preabsorbed pooled sera. Positive recombinant phage clones from tertiary screenings were subjected to pBlueScript SK plasmid excision using helper phage, and recombinant plasmids were DNA sequenced using the forward T3 primer (Stratagene manual).

Cloning and expression of *M. tuberculosis* antigens. Synthetic oligonucleotides were designed for PCR and cloning of the B.4, B.6, B.10, B.M, and C17 genes, including a known 38-kDa antigen (2). *M. tuberculosis* genomic DNA was extracted as previously described with a few modifications (7). The PCR mixture contained 0.1 μg of *M. tuberculosis* genomic DNA, 50 pmol of synthetic oligonucleotides (Genset, Singapore), 10 μl of GC-melt (from the Advantage-GC Genomic PCR kit [Clontech, Inc., Palo Alto, Calif.], 5 μl of 10× Expand High-Fidelity DNA Polymerase buffer (containing $MgCl_2$), 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.75 U of Expand High-Fidelity DNA Polymerase (Boehringer GmbH, Mannheim, Germany), and distilled H_2O to a total reaction volume of 50 μl. PCR amplications were carried out in a DNA PTC-100 thermal cycler (MJ Research, Watertown, Mass.) under the following conditions: 94°C for 2 min; 9 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min; 19 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min plus 20 s per cycle; and a final extension step at 72°C for 7 min.

PCR products were initially cloned into pGEM-T Easy vector (Promega, Madison, Wis.) and subsequently subcloned into pQE30 expression vector (Qiagen, Inc., Valencia, Calif.). DH5α and Epicurian Coli XL10-Gold (Stratagene) *E. coli* cells were used for cloning and maintenance of plasmid DNA vector and recombinant plasmid DNA, whereas M_{15} *E. coli* cells (Qiagen) were used for expression studies. Transformation of plasmid DNA into *E. coli* cells was done by a heat shock method as described by Crouse et al. (13). Plasmid DNA extraction was performed using the Wizard Midi and Miniprep DNA purification systems (Promega). DNA sequencing was carried out on an Automated DNA Sequencer, model 373A (Perkin-Elmer Applied Biosystems, Foster City, Calif.) using the forward (5′-GAAT[redacted]CATTAAAGAGGAGAAA-3′) and reverse (5′-GTTCTG AGGTCATTACT[redacted]G-3′) primers of the pQE30 vector and primers synthesized based on internal sequences obtained from DNA sequencing of the TB genes. Recombinant proteins were expressed as NH_2-terminally polyhistidine-tagged fusion proteins and purified from the M_{15} *E. coli* cell extracts to near-homogeneity by Ni-nitrilotriacetic acid (NTA) affinity chromatography (Qiagen, The QIAexpressionist, 3rd ed., July 1997). Quantitation of recombinant proteins was carried out using the *DC* Protein Assay Kit (Bio-Rad). SDS-PAGE gels for visualization were stained either with Coomassie brilliant blue R-250 or with silver stain (27).

SDS-PAGE and Western blotting of *M. tuberculosis* recombinant antigens. To maintain consistency, Tris-HCl two-dimensional (2D) preparative ready gels (Bio-Rad) were used for Western blotting. A total of 10 μg of purified recombinant antigen was subjected to SDS-PAGE and Western blotted onto Hybond-C nitrocellulose membranes (Amersham) using the Bio-Rad TransBlotter (according to the manufacturer's protocol). After transfer, the membrane was blocked in 5% skim milk-TBST, air dried, and stored at 4°C until further use.

Immunoblot analysis of recombinant antigens. Each membrane containing Western-blotted antigen was cut into strips of 3-mm width (a total of 23 strips from each blot), and each strip was used for screening with a serum specimen. One strip was used as an internal positive control probed with a positive serum specimen that is reactive to the recombinant protein antigens. A second strip was probed with the commercially available anti-RGS His probe (Qiagen). Screening was carried out by incubating each strip in trays with 1 ml of diluted serum specimen (1:100 in 1% skim milk-TBST) per well for 1 h with rocking at room temperature. The strips were then washed four times in TBST, followed by incubation with alkaline phosphatase-conjugated goat anti-human Ig (Harlan Sera Lab) for 1 h with rocking at room temperature. The strips were again washed four times in TBST and then allowed to develop in 1 ml of NBT-BCIP substrate (Bio-Rad) for 4 min. The reaction was stopped by washing four times in distilled H_2O.

Western blot scoring. The reactivities of recombinant proteins to serum specimens were interpreted based on the intensities of bands obtained on an X-Rite *400* densitometer (X-Rite Inc., Grandville, Mich.). The cutoff values (expressed as densities) for each individual recombinant antigen were determined based on the range observed for normal serum specimens.

Serum specimens. A total of 139 human sera were used in this study, of which 119 serum specimens were purchased from BioClinical Partners, Inc., Franklin, Mass., whereas 20 were donated by healthy laboratory workers. The control groups consisted of two panels, (i) a panel of 50 serum specimens from healthy individuals, of which 20 (laboratory workers) had been *Mycobacterium bovis* BCG vaccinated previously and 30 (BioClinical) had unknown BCG status, and (ii) a panel of 19 serum specimens (BioClinical) from individuals with non-TB respiratory disease (lung cancer).

The test group consisted of 48 serum specimens (BioClinical) from bacteriologically confirmed active TB patients and 22 serum specimens (BioClinical) from patients with active TB. The active-TB serum specimens comprised 28 from pulmonary-TB and 20 from extrapulmonary-TB patients. Serum specimens from the inactive-TB panel were from patients with positive PPD skin tests but negative acid-fast stains of sputum and bacterial culture. All sera were aliquoted and stored at −70°C before use.

TABLE 1. Results of homology searches against the GenBank protein sequence databases[a]

Relative molecular size (kDa)	N-terminal sequence	Match (NBCI)[b]
~58	SKLIEYDELALEAME	db: $_{2}$SKLIEYDETARRAME$_{16}$; 55.7 kDa; GroEL1/protein Cpn60 (18, 30); pID = g44601; X60350 (80% match)
~48	AEVDAYKFDPDAVD	db: $_{161}$AEFDAYRRDPMA$_{172}$; probable exported protease, has signal sequence, very similar to three proteases/peptidases from *Streptomyces*; pID = e235164; MTCY427.04c (51% match)
~34	MEIDILAVAAP	db: $_{117}$IEVDLLDLDAP$_{127}$; 33 kDa; mycocerosic acid synthase; pID = g149978; M95808 (56.9% match)
~14	ATTLPVQRHDARL	db:ATTLPVQRHPRSL; 14/16 kDa (21); pID = g244562; M76712 (69.0% match)

[a] Proteins showing the highest homology to the *M. tuberculosis* proteins excised for N-terminal sequencing are shown.
[b] db, database; pID, protein identification.

Databases and software. Nucleotide and protein sequence analysis was carried out using the basic BLAST 2.0 search program from the National Center for Biotechnology Information (NBCI) and the *M. tuberculosis* BLAST server at the Sanger Centre (Cambridge, United Kingdom).

Statistical analysis. Sensitivities, specificities, and positive and negative predictive values were calculated using the Win Episcope 1.0 (Borland International Inc.).

RESULTS

Identification and isolation of *M. tuberculosis* antigens. Immunoblot analysis of *M. tuberculosis* total proteins revealed protein bands which reacted with the pooled active sera but not with the pooled normal sera. When the respective bands were concentrated on a long stacking gel, excised, and Western blotted, these bands were reactive with the pooled active sera but not with pooled normal sera, thus confirming the authenticity of these excised proteins as those initially observed in the primary screening (Fig. 1). These proteins were identified by homology searches against protein sequence databases, which gave a high percentage of homology to *Mycobacterium* proteins (Table 1). PCR primers were designed to isolate and clone the genes coding for four proteins (B.4, B.6, B.10, and B.M) which gave the highest matches based on a BLAST homology search against the SwissProt database.

Concurrently, primary screening of the phage expression library gave eight reactive phage recombinants, of which six were further confirmed positive by secondary and tertiary screenings (Fig. 2). These clones were subjected to plasmid DNA excision, and restriction enzyme digestions with *Eco*RI indicated that all the clones contained a 2-kb insert. DNA sequencing revealed that all the clones were identical, having a 1.161-kb open reading frame (in frame with the vector's ATG initiation codon) which coded for a proline-rich protein. A summary of all five TB antigens, with the respective gene sizes, theoretical molecular masses, and pI values is shown in Table 2.

Expression and purification of recombinant antigens. Expression was detected by probing immunoblots containing these antigens using the commercial anti-RGS His antibody. The levels of expression observed were high for the B.4, B.M, and 38-kDa proteins, moderate for the B.6 and B.10 proteins, and low for the C17 protein (Fig. 3). All of these recombinant proteins, except for C17, were present in the insoluble fraction of an SDS-PAGE analysis (data not shown), indicating that these proteins formed inclusion bodies and were insoluble. As such, these recombinant proteins were purified by Ni-NTA affinity chromatography in 8 M urea, and the SDS-PAGE profile of the purified antigens subsequently used for immunoblots is shown in Fig. 4. The approximate yields of recombinant antigens purified through Ni-NTA were 36 mg/liter for B.4, 0.5 mg/liter for B.6, 0.2 mg/liter for B.10, 15 mg/liter for B.M, <0.1 mg/liter for C17, and 10 mg/liter for the 38-kDa protein.

Reactivities of the recombinant antigens to TB serum specimens. The different reactivities of recombinant TB antigens on immunoblot strips probed with serum specimens and anti-RGS His are shown in Fig. 5. The respective cutoff values for determining reactivity to the different recombinant antigens were obtained based on the mean densities observed in sera from the control group of healthy individuals (n = 50). The cutoff values were densities of >0.04 for the B.4 and B.6 bands, ≥0.04 for the B.10 and B.M bands, ≥0.15 for the C17 band, and >0.15 for the 38-kDa band.

Based on the Western blot assay, the reactivities of these antigens to a panel of active-TB serum specimens are shown in Table 3. Percentages of reactivities and positive and negative predictive values for each antigen were calculated based on sera from infected individuals (n = 48; pulmonary and extrapulmonary TB) and sera from the control group of healthy individuals (n = 50), with a P value of <0.05. The specificities for the B.4, B.6, B.10, B.M, C17, and 38-kDa recombinant antigens are 94, 88, 100, 96, 90, and 98%, respectively. All of the recombinant TB antigens showed substantial reactivity to

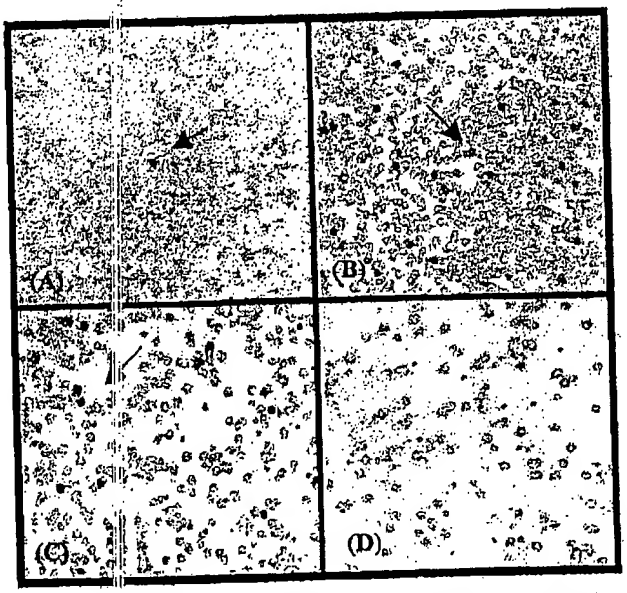

FIG. 2. Screening of the lambda ZAP Phage expression library of *Eco*RI-restricted genomic DNA of *M. tuberculosis*, using pooled human sera from individuals with active TB. (A) Primary screening; (B and C) secondary screening; (D) negative control consisting of plaques of nonrecombinant phages. Arrows indicate plaques containing positive recombinant phage.

TABLE 2. TB antigens genes which were cloned and expressed in pQE30[a]

Antigen	Size of gene (kb)	Theoretical[b]: Molecular mass (kDa)	Theoretical[b]: pI
B.4	1.617	55.8	5.12
B.6	1.560	55.0	5.03
B.10	0.903	32.9	4.95
B.M	0.432	16.1	5.00
C17	1.161	37.5	9.43

[a] The resultant recombinant proteins will be approximately 1.4 to 1.5 kDa larger than the theoretical molecular mass shown, due to the histidine tag at the N terminus.

[b] Obtained using the software "Compute pI/Mwt" from the ExPASy home page, Swiss Institute of Bioinformatics, Geneva, Switzerland.

active-TB specimens, both pulmonary and extrapulmonary. The B.4 antigen was reactive with 58.3% of the active-TB panel, compared to 37.5% detected by the known 38-kDa antigen. In addition, the B.4 antigen showed reactivity to 27.3% of the inactive-TB specimens compared to other TB antigens, which exhibited lower percentages of reactivity to these specimens (Table 3). The B.6 antigen was found to exhibit specific reactivity to pulmonary-TB specimens (46.4%) compared to extrapulmonary specimens (5%) (Fig. 6). All the other antigens were able to detect antibodies in both pulmonary- and extrapulmonary-TB specimens.

DISCUSSION

A number of *M. tuberculosis* antigens have been identified and characterized by various methods employing polyclonal antibodies from rabbits or monoclonal antibodies (MAbs) from hybridomas generated from immunized mice. Such antibodies were used widely for identification and purification of protein antigens by affinity chromatography (22), immunoscreening of clones from DNA libraries of *M. tuberculosis* (24, 37), and analyses of total-cell lysates or secretory proteins from culture medium by both one- and two-dimensional gel electrophoresis (16, 32). Immunogenicity in animals (e.g., mice or rabbits), however, may not reflect relevance to human immune responses. Thus, attempts were made to search for candidate serodiagnostic antigens by directly testing mycobacterial proteins with tuberculous-patient sera on immunoblots of one-

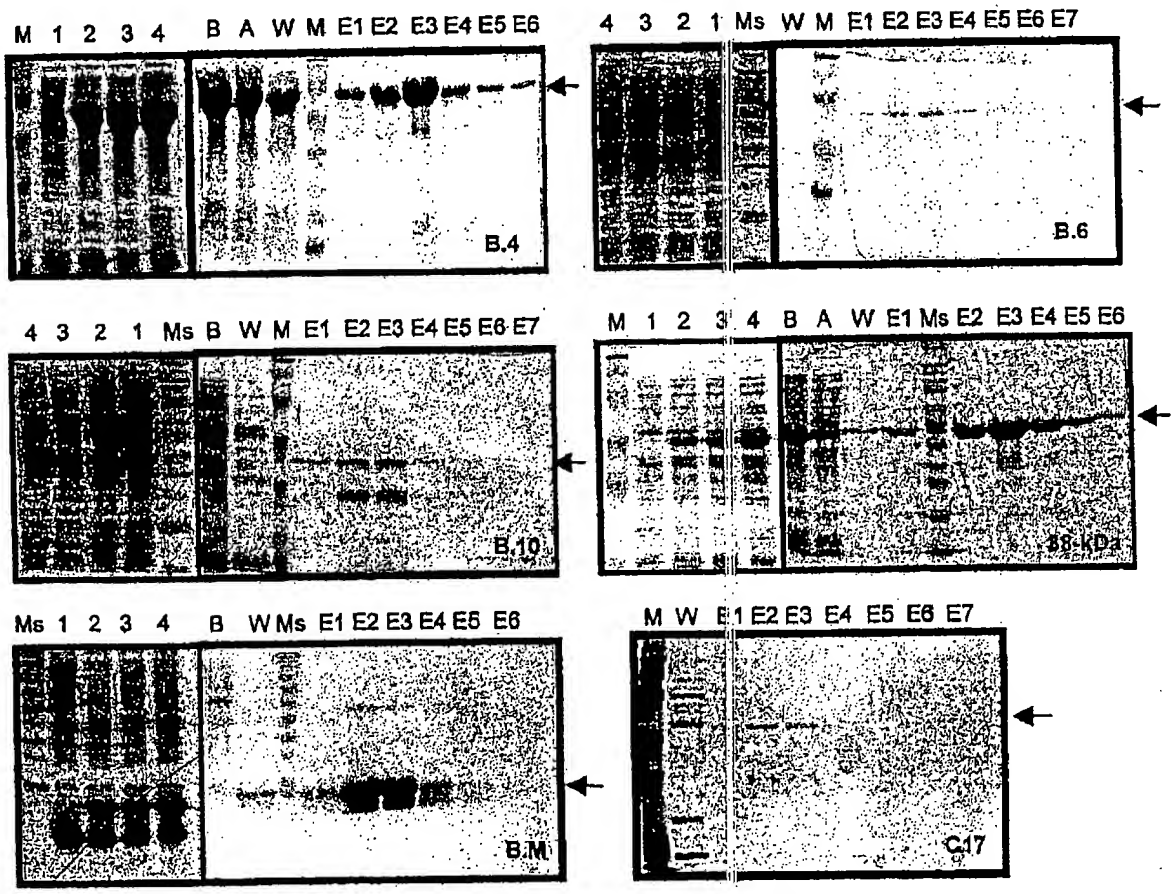

FIG. 3. Expression and affinity chromatography purification profiles of the *M. tuberculosis* antigens expressed in 100 ml of M_{15}/*E. coli* cultures. Lanes: M and Ms, protein molecular weight markers (M, Kaleidoscope standards; Ms, Sigma Broad range); 1 through 4, aliquots taken at 0, 1, 2, and 3 h, respectively, after induction with 1 mM IPTG; B, total cell lysate before passing through Ni-NTA column; A, total lysate after passing through column; W, wash fractions in 8 M urea buffer (pH 6.5 to 5.9); E1 to E7, eluted fractions in 8 M urea buffer (pH 4.5). The bulk of the recombinant proteins were observed to be eluted in fractions in 8 M urea buffer (pH 4.5). The bulk of the recombinant proteins were observed to be eluted in fractions E2 and E3 (arrows). All the gels were stained with Coomassie brilliant blue, except for C17, which was silver stained.

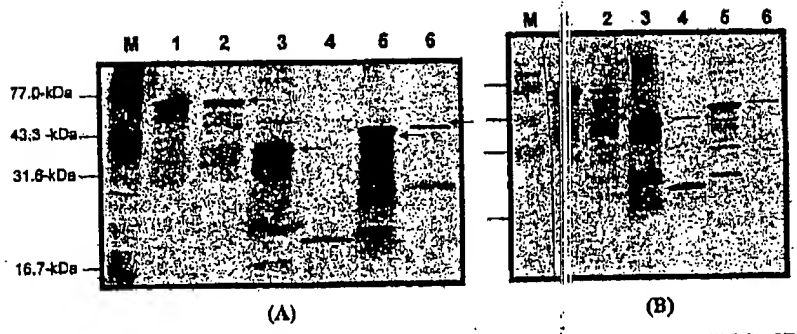

FIG. 4. Recombinant *M. tuberculosis* antigens (arrows) purified by Ni-NTA affinity chromatography is observed on an SDS-PAGE gel (silver stained) (A) and on a Western blot of a duplicate gel which was probed with anti-RGS His antibody followed by detection with alkaline phosphatase-conjugated goat anti-mouse Ig and NBT-BCIP substrate (B). Some truncated products of the recombinant proteins were observed which were detected by the antibody. Lanes: M, Kaleidoscope prestained standards; 1, B.4; 2, B.6; 3, B.10; 4, B.M; 5, 38-kDa protein; 6, C17.

dimensional and two-dimensional separations of antigenic extracts or culture filtrates of *M. tuberculosis* H37Rv (4, 26).

In this study, we used antibodies present in the sera of infected individuals to screen total-cell lysates and a phage expression library of *M. tuberculosis* DNA. To date, there is no single immunodominant species-specific antigen for detection of tuberculosis. We have chosen to use pooled sera from several infected individuals to allow identification of several immunoreactive antigens reactive to antibodies present in each serum. In addition, the *M. tuberculosis* genome database completed by the Sanger Centre (12) allowed for the rapid identification of these immunoreactive antigens by homology searches against available protein and gene databases, which also facilitated the identification of these gene sequences for cloning.

We have successfully identified and characterized five antigens using a Western blot total-cell-lysate approach; of these, the B.6, B.10, and C17 antigens are novel and showed high degrees of nucleotide identity to unpublished *M. tuberculosis* H37Rv genes. Based on DNA sequencing results, the B.6 antigen gene was found to have 99% nucleotide identity to a gene coding for a protein with homology to exported proteases or peptidases. The B.10 antigen exhibited 99 and 98% nucleotide identities to the *M. bovis* acyl coenzyme A (CoA) synthase (accession no. U75685) and mycocerosic acid synthase (accession no. M95[illegible]08) genes, respectively. The C17 antigen exhibited 99 to 100% nucleotide identity to a gene coding for PE-PGRS (polymorphic GC-rich repetitive sequence) proteins, a member of PE (proline-glutamic acid) families of clustered genes coding for glycine-rich proteins which may have immunological and pathogenic implications (12).

The B.4 antigen exhibited 99.8% amino acid and 98.9% nucleotide sequence homology to the Cpn-60 protein reported by Kong et al. (18). The diagnostic potential of a 65-kDa protein (also a Cpn-60 family of heat shock proteins) by both serological and PCR methods has been demonstrated (28). The B.M antigen has 99% nucleotide identity to the reported *M. tuberculosis* 14-kDa antigen gene (accession no. M76712) and the gene for the 19-kDa major membrane protein purified from the virulent Erdman strain of *M. tuberculosis* (21). The serological value of this 19-kDa antigen was shown by 85% reactivity to a panel of 56 sera from individuals with active pulmonary TB (9).

Antigens of diagnostic importance for *M. tuberculosis* identified to date include the 65-, 45-, 30/31-, 19-, and 12-kDa proteins and the 38-kDa lipoprotein (9, 11, 14, 36). Among

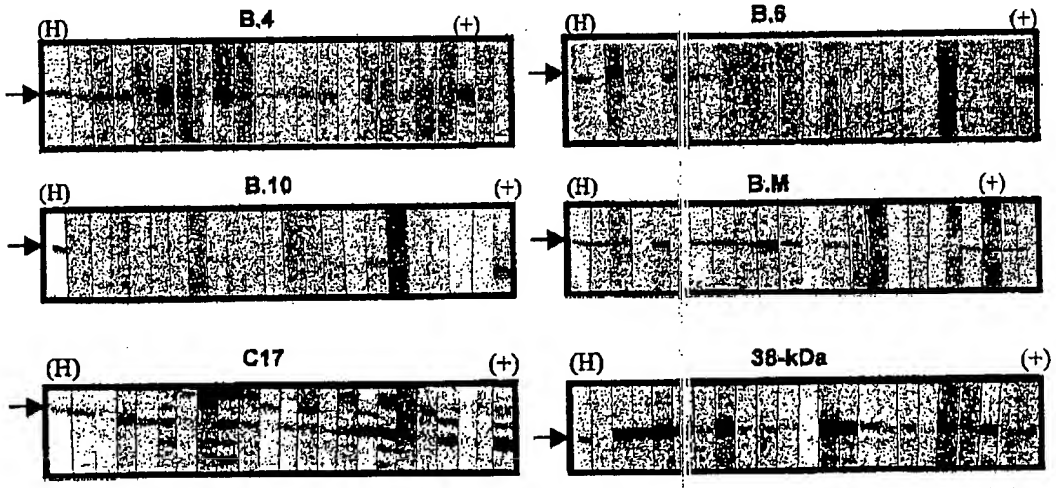

FIG. 5. Immunoscreening of recombinant TB antigens by a Western blot assay. Arrows indicate the positions of recombinant antigens on the immunoblots. Each strip was probed with different serum specimens. A strip was probed with anti-RGS His (H) to indicate the position of protein on the blots. The positive control (+) is represented by a strip probed with a known serum specimen which is reactive to the specific recombinant antigen.

TABLE 3. Reactivities and positive and negative predictive values of recombinant TB antigens against different sera panels

Antigen[a]	Reactivity (%) of sera from:				PPV[b] (%)	NPV[c] (%)
	Healthy controls (n = 50)	Active-TB patients (n = 48)	Inactive-TB patients (n = 22)	Lung cancer patients (n = 19)		
B.4	6.0	58.3	27.3	10.5	90.3	70.2
B.6	12.0	29.2	9.1	5.3	70.0	56.4
B.10	0	35.4	0	5.3	100.0	61.7
B.M	4.0	20.8	4.6	0	83.3	55.8
C17	10.0	22.9	0	0	68.8	54.9
38-kDa antigen	2.0	37.5	9.1	10.5	96.6	71.0

[a] A known 38-kDa antigen (GenBank accession no. M30046 [2]) of *M. tuberculosis* was included in the screening.
[b] PPV, positive predictive value, $P > 0.05$.
[c] NPV, negative predictive value, $P > 0.05$.

these, the 38-kDa protein was shown to be the most specific and sensitive for detecting antibodies against *M. tuberculosis* and is specific for TB complex species (35). As such, we have chosen to clone and express this antigen to be used in immunoscreening against the serum panels for comparison with our recombinant TB antigens. This 38-kDa antigen was expressed as an insoluble protein in the pQE30/*E. coli* system; similarly, Singh et al. reported the expression of this 38-kDa antigen as an insoluble unfused protein in *E. coli* (31).

We have chosen the Qiagen expression system for cloning and expression of the TB antigens. Each expressed recombinant protein contained a nonimmunogenic 6× His tag at the N terminus which could be immunodetected by anti-RGS His. A substantially low expression level was observed for the C17 protein. This may be due to the codon usage of this protein, which is rich in proline (46.6%), as it is reported that the expression level of a gene decreases with an increase in the use of rare codons (17). In addition, the Kyte-Doolittle hydropathy plot revealed that it is very hydrophilic, which explains the soluble nature of this protein (19). As most of the recombinant antigens were insoluble, we have chosen a Western blot assay for preliminary screening against serum specimens.

Immunoblot assays using human sera were described previously for analysis of HPLC-purified 45/47-kDa antigen complex (15). Rovatti et al. reported a semiquantitative Western blot serological test to identify PPD-positive individuals, using a discriminat[illegible] score for the *M. bovis* BCG antigen complex A60 against M[illegible] Abs (25). In our immunoblot assay system, we used affinity-p[illegible]rified recombinant proteins to detect antibodies in serum spe[illegible]imens and have included the known 38-kDa lipoprotein a[illegible] a control for our purification and Western blot assay system. [illegible]Zhou et al. reported the use of this antigen in a rapid membr[illegible]ne-based assay that gave a specificity of 92%, very close to [illegible]our in-house immunoblot assay of the 38-kDa protein, whic[illegible] gave 98% specificity (38). The 38-kDa antigen was included [illegible]n the assay system as a further control for specificity and sen[illegible]sitivity. This was further compared to a commercially availabl[illegible] diagnostic test kit which uses two antigens, one of which is t[illegible]e 38-kDa antigen. All 13 of the 18 active-TB serum specim[illegible]ens that tested positive with the 38-kDa protein in our Weste[illegible]n blot assay also tested positive with the kit. The percentage o[illegible] specificity of the 38-kDa antigen in this assay is comparable t[illegible] that with the kit (98 and 100%, respectively).

Data anal[illegible]sis from our assay system indicated that the B.4, B.6, B.10, B.[illegible]M, C17, and 38-kDa recombinant antigens reacted with antibod[illegible]s in serum specimens of TB-infected individuals. The reactiviti[illegible]es were sufficiently differentiated from those of serum specim[illegible]ens from healthy individuals and from individuals with inactive TB or lung cancer. Screening of the same serum panels using two commercially available TB diagnostic kits of high specificity indicated the presence of immunologically specific antibod[illegible]es reacting to these TB antigens (submitted for publication). Reports have also shown that other recombinant antigens did exhibit low levels of cross-reactivities to sera from healthy individuals, which may be due to cross-reactive epitopes or analogue[illegible] to other bacterial proteins (8). To circumvent this problem, thi[illegible] use of MAbs with recombinant antigens in competition with patient sera as a test assay was reported to give a high degree of specificity (10). Alternatively, we have to further optimize the immunoblot assay to decrease cross-reactivities.

In conclusion, we have demonstrated the identification and cloning of five TB antigen genes for expression in an *E. coli* system and shown the potential of each recombinant antigen as a serodiagnostic marker for detection of TB infections. We are in the process of validating the diagnostic utility of these antigens in various test formats.

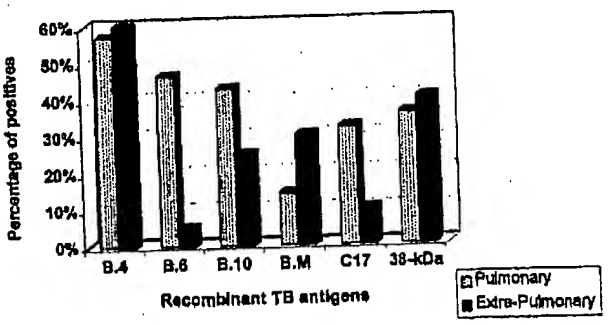

FIG. 6. Graph showing percentages of reactivity for the recombinant TB antigens against the pulmonary-TB (n = 28) and extrapulmonary-TB (n = 20) serum specimens. The B.6 antigen detected antibodies in 46.4% of the pulmonary-TB compared to only 5% of the extrapulmonary-TB serum specimens. The rest of the TB antigens did not exhibit such significant differentiation of reactivity between the two serum panels.

ACKNOWLEDGMENTS

We are gr[illegible]teful to Christopher Froggatt, Kean Chong Loh, Cindy Luo, and Ad[illegible]an Lee at the Fermentation and Purification Laboratory of BTC for g[illegible]nerously helping us to produce and purify the recombinant antigen[illegible] for screenings.

REFERENCES

1. Amicosante, M., S. Barnini, V. Corsini, G. Paone, C. A. Read, Jr., P. L. Tartoni, M. Singh, C. Abera, A. Bisetti, S. Senesi, M. Campa, and C. Saltini. 1995. Evaluation of a novel tuberculosis complex-specific 34-kDa protein in the serological diagnosis of tuberculosis. Eur. Respir. J. 8:2008–2014.
2. Andersen, A. B., and E. B. Hansen. 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. 57:2481–2488.
3. Banica, D. G. Algeorge, A. Motoiu, A. Petre, and M. Toanca. 1994. The possibilities for improving the serological diagnosis of active tuberculosis by using new mycobacterial antigens and immunoblot and ELISA technique. Pneumoftiologia 43:173–177.
4. Bassey, E. O. E., D. Catty, D. S. Kumararatne, and C. Raykundalia. 1996. Candidate antigens for improved serodiagnosis of tuberculosis. Tubercle Lung Dis. 77:136–145.
5. Beige, J., J. Lokies, T. Schaberg, U. Finckh, M. Fischer, H. Mauch, H. Lode, B. Kobler, and A. Rolfs. 1995. Clinical evaluation of a *Mycobacterium tuberculosis* PCR assay. J. Clin. Microbiol. 33:90–95.
6. Bloom, B. R., and C. J. Murray. 1992. Tuberculosis: commentary on a reemergent killer. Science 257:1055–1064.
7. Bose, M., A. Chander, and R. H. Das. 1993. A rapid and gentle method for the isolation of genomic DNA from mycobacteria. Nucleic Acids Res. 21: 2529–2531.

8. Bothamley, G. H. 1995. Serological diagnosis of tuberculosis. Eur. Respir. J. Suppl. 20:676S–688S.
9. Bothamley, G., H. Batra, V. Ramesh, A. Chandramul, and J. Ivanyi. 1992. Serodiagnosis value of the 19-kilodalton antigen of *Mycobacterium tuberculosis* in Indian patients. Eur. J. Clin. Microbiol. Infect. Dis. 11:912–915.
10. Bothamley, G. H., and R. M. Rudd. 1994. Clinical evaluation of a serological assay using a monoclonal antibody (TB72) to the 38-kDa antigen of *M. tuberculosis*. Eur. Respir. J. 7:240–246.
11. Coates, A. R., H. Nicolai, M. J. Pullen, A. Guy, S. D. Chaparas, and D. A. Mitchison. 1989. The 45-kDa molecule of *Mycobacterium tuberculosis* identified by immunoblotting and antibodies as antigenic in patients with tuberculosis. Br. J. Exp. Pathol. 70:215–225.
12. Cole, S. T., R. Brosch, J. Parkhill, and T. Garnier. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544.
13. Crouse, G. F., A. Frishauf, and H. Lehrach. 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. 101:78–79.
14. Deshpande, E. R., M. B. Khan, and R. G. Navalkar. 1993. Immunological evaluation of a 12-kDa protein of *Mycobacterium tuberculosis* by enzyme-linked immunosorbent assay. Tubercle Lung Dis. 74:382–387.
15. Dingbouga, S., F. Fumoux, A. Zoubga, P. T. Sanou, and G. Marchal. 1996. Immunoblot analysis for serodiagnosis of tuberculosis using a 45/47-kilodalton antigen complex of *Mycobacterium tuberculosis*. Eur. Respir. J. 9:288–292.
16. Espitia, C., R. Espinosa, R. Saavedra, R. Mancilla, F. Romain, A. Laqueyrerie, and C. Moreno. 1995. Antigenic and structural similarities between *Mycobacterium tuberculosis* 50- to 55-kilodalton and *Mycobacterium bovis* BCG 45- to 47-kilodalton antigens. Infect. Immun. 63:580–584.
17. Grantham, R., C. Gautier, M. Gouy, M. Jacobzone, and R. Mercier. Codon catalog usage is a genome strategy modulated for gene expressivity. Nucleic Acids Res. 9:43r–74r.
18. Kong, T. H., A. R. Coates, P. D. Butcher, C. J. Hickman, and T. M. Shinnick. 1993. *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. Proc. Natl. Acad. Sci. USA 90:2608–2612.
19. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105–132.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
21. Lee, B. Y., S. A. Hefta, and P. J. Brennan. 1992. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. Infect. Immun. 60:2066–2074.
22. Ljungqvist, L., A. B. Andersen, P. Andersen, K. Haslov, A. Worsaae, J. Bennedsen, and I. Heron. 1990. Affinity purification, biological characterization and serological evaluation of defined antigens from *Mycobacterium tuberculosis*. Trop. Med. Parasitol. 41:333–335.
23. Lyashchenko, K., R. Colangeli, M. Houde, H. A. Jahdali, D. Menzies, and M. L. Gennaro. 1998. Heterogeneous antibody responses in tuberculosis. Infect. Immun. 66:3936–3940.
24. Parra, C. A., L. P. Londono, P. D. Portillo, and M. E. Patarroyo. 1991. Isolation, characterization, and molecular cloning of a specific *Mycobacterium tuberculosis* antigen gene: identification of a species-specific sequence. Infect. Immun. 59:3411–3417.
25. Rovatti, E., M. P. Corradi, M. Amicosante, P. L. Tartoni, W. Panini, A. Ancora, A. M. Cenci, L. Zucchi, L. Monno, G. Angarano, and C. Saltini. 1995. Evaluation of a Western blot serum test for the diagnosis of *Mycobacterium tuberculosis* infection. J. Commun. Dis. 27:208–214.
26. Samanich, K. M., J. T. Belisle, M. G. Sonnenberg, M. A. Keen, S. Zolla-Pazner, and S. Laal. 1998. Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*. J. Infect. Dis. 178: 1534–1538.
27. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68:850–858.
28. Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. 169:1080–1088.
29. Shinnick, T. M., and R. C. Good. 1995. Diagnostic mycobacteriology laboratory practice. Clin. Infect. Dis. 21:291–299.
30. Shinnick, T. M., M. H. Vodkin, and J. C. Williams. 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. 56:446–451.
31. Singh, M., A. B. Andersen, J. E. G. McCarthy, M. Rohde, H. Schulte, E. Sanders, and I. N. Timmis. 1992. The *Mycobacterium tuberculosis* 38-kDa antigen: overproduction in *Escherichia coli*, purification and characterization. Gene 117:53–60.
32. Sonnenberg, M. G., and J. T. Belisle. 1997. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect. Immun. 65:4515–4524.
33. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354.
34. World Health Organization. 1999. The World Health Report. World Health Organization, Geneva, Switzerland.
35. Young, D., L. Kent, A. Rees, J. Lamb, and J. Ivanyi. 1986. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. 54:177–183.
36. Young, D. B. 1988. Structure of mycobacterial antigens. Br. Med. J. 44:562–583.
37. Young, R. A., B. R. Bloom, C. M. Grossklnsky, J. Ivanji, D. Thomas, and R. W. Davies. 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA 82:2583–2587.
38. Zhou, A. T., W. L. Ma, P. Y. Zhang, and R. A. Cole. 1996. Detection of pulmonary and extrapulmonary tuberculosis patients with the 38-kilodalton antigen from *Mycobacterium tuberculosis* in a rapid membrane-based assay. Clin. Diagn. Lab. Immunol. 3:337–341.